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Article

Identification of Distinct Ligands for the C-type Lectin Receptors Mincle and Dectin-2 in the Pathogenic Fungus *Malassezia*

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SUMMARY

Various C-type lectin receptors (CLRs), including Mincle and Dectin-2, function as pattern recognition receptors and play a central role in immunity to fungal pathogens. However, the precise structures of the CLR ligands in various pathogenic fungi have yet to be completely defined. Here we report that *Malassezia*, an opportunistic skin fungal pathogen, is cooperatively recognized by Mincle and Dectin-2 through distinct ligands. Solvent-based fractionation revealed that Mincle and Dectin-2 recognize lipophilic and hydrophilic components of *Malassezia*, respectively. Mass spectrometry and nuclear magnetic resonance (NMR) revealed glyceroglycolipid and unique mannosyl fatty acids linked to mannitol as two Mincle ligands. An *O*-linked mannobiose-rich glycoprotein was identified as a *Malassezia* ligand for Dectin-2. Cytokine production in response to the Mincle ligands and the Dectin-2 ligand was abrogated in Mincle$^{-/-}$ and Dectin-2$^{-/-}$ dendritic cells, respectively. These results demonstrate that Mincle and Dectin-2 recognize distinct ligands in *Malassezia* to induce host immune responses.

INTRODUCTION

Innate host response is mediated by pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs) including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and C-type lectin receptors (CLRs) (Robinson et al., 2006; Takeuchi and Akira, 2010). Among these PRRs, CLRs play a crucial role in recognizing the complex structures, which are composed of carbohydrate residues, of various fungal pathogens (Hardison and Brown, 2012; Robinson et al., 2006). Recently, some CLRs have also been demonstrated to directly transduce the signals to produce inflammatory cytokines through signaling subunits such as FcRγ (Robinson et al., 2006); however, the ligands of most CLRs remain largely unclear.

Mincle (also called Clec4e or Clecsf9) is a CLR expressed in activated macrophages and dendritic cells (DCs) subjected to several types of stress (Matsumoto et al., 1999). We have previously shown that Mincle is an activating receptor coupled with the Fc receptor γ (FcRγ) chain, an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor (Yamasaki et al., 2008). We found that Mincle recognizes mycobacteria and identified trehalose 6,6′-dimycolate (TDM; cord factor) as a specific ligand (Ishikawa et al., 2009). TDM is a glycolipid derived from the mycobacterial cell wall and has been shown to possess potent adjuvant activity (Hunter et al., 2006). Although the TDM expression is restricted to mycobacteria, corynebacteria, and *Nocardia*, we have recently found that Mincle also recognizes *Malassezia* species (Yamasaki et al., 2009). However, the Mincle ligand in *Malassezia* has not yet been identified, thus suggesting the existence of a ligand other than TDM in this fungus.

Fungi of the *Malassezia* genus are found in the normal flora of human skin. These species are considered to be harmless commensal organisms under normal circumstances; however, they are also widely known as opportunistic pathogens (Gaitanis et al., 2012; Guillot and Bond, 1999). They have been reported to be associated with diverse dermatological pathologies including pityriasis versicolor, seborrheic dermatitis, atopic dermatitis, and folliculitis (Ashbee and Evans, 2002; Guillot and Bond, 1999). *Malassezia* species also cause lethal systemic infections in newborn infants receiving intravenous lipid emulsions (Marcon

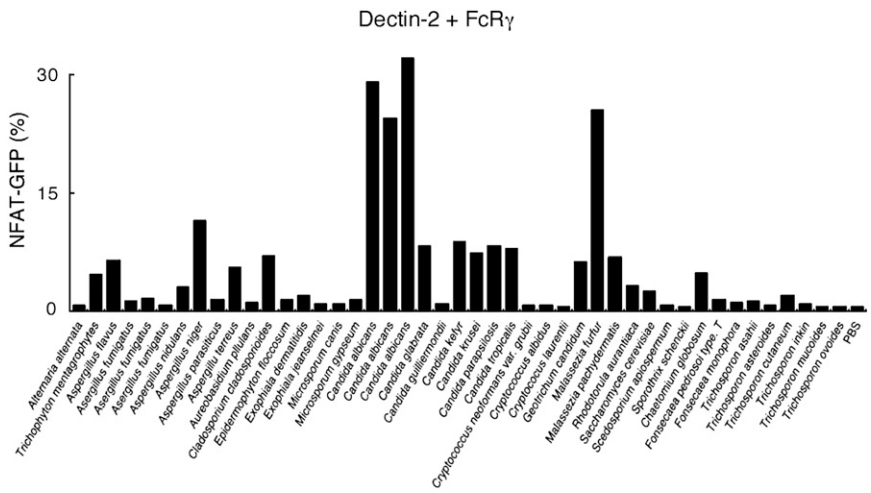

Figure 1. Dectin-2 Recognizes *Malassezia* Species

Screening of pathogenic fungi for Dectin-2 ligand activities. The reporter cell lines expressing Dectin-2 + FcRγ were cocultured with the indicated pathogenic fungi for 18 hr. The NFAT-GFP induction was analyzed by flow cytometry (See also Figure S1). Representative results from two independent experiments with similar results are shown.

and Powell, 1992; Redline and Dahms, 1981). Intriguingly, *Malassezia* species are unique among other fungi in that they require lipid for their growth (Schmidt, 1997). However, the mechanism underlying the recognition of *Malassezia* by host cells has not been fully elucidated.

Dectin-2 (also called Clec4n) is another FcRγ-coupled CLR that is constitutively expressed on DCs, tissue macrophages, and inflammatory monocytes (Sato et al., 2006; Taylor et al., 2005). Dectin-2 is reported to recognize a variety of fungi including *Candida albicans*, *Saccharomyces cerevisiae*, *Histoplasma capsulatum*, *Microsporum audouinii*, and *Trichophyton rubrum* (McGreal et al., 2006; Ritter et al., 2010; Sato et al., 2006). It is therefore possible that Dectin-2 also recognizes *Malassezia*. Dectin-2 is reported to bind to high-mannose structures (McGreal et al., 2006; Sato et al., 2006), especially mannans in *C. albicans* (Saijo et al., 2010); however, the precise structure of the Dectin-2 ligand has not yet been fully defined.

In this study, we demonstrate that two CLRs, Mincle and Dectin-2, cooperatively induce the immune response to the same fungus, *Malassezia*, through the recognition of distinct ligands.

RESULTS

Dectin-2 Recognizes *Malassezia* Species

We first tried to compare the recognition property of Mincle and Dectin-2. More than 45 species of pathogenic fungi were analyzed by using NFAT-GFP (nuclear factor of activated T cells-green fluorescent protein) reporter cells expressing FcRγ with Mincle or Dectin-2. As we have previously reported, Mincle selectively recognizes *Malassezia* species (Figure S1A) (Yamasaki et al., 2009). In sharp contrast, Dectin-2 broadly recognizes a variety of fungi including *Trichophyton*, *Aspergillus*, *Cladosporium*, *Candida*, and *Malassezia* species (Figure 1). Interestingly, only *Malassezia* species represented an overlapping target for both receptors. We confirmed that *Malassezia* could not activate reporter cells expressing FcRγ alone (data not shown).

Mincle and Dectin-2 Recognize *Malassezia* by Distinct Mechanisms

In order to identify the ligand(s) for Mincle and Dectin-2 in *Malassezia*, we tried to extract active fraction with various aqueous-organic solvents (Figure 2A). These extracts and solvent-treated fungal cells were then tested to determine their ability to stimulate NFAT reporter cells. We found that *M. pachydermatis* treated with chloroform:methanol (C:M) selectively lost their Mincle-stimulating activity (Figure 2B). Simultaneously, we analyzed the activity of extracted fractions in plate-coated form and found that only the C:M phase after C:M extraction showed strong stimulatory activity (Figure 2C). These findings suggested that Mincle recognizes some lipophilic component(s) in *Malassezia*.

On the other hand, the activity for Dectin-2 was efficiently extracted into the water phase, indicating that the Dectin-2 recognizes hydrophilic component(s) in *Malassezia* (Figures 2D and 2E). These results suggest that *Malassezia* may possess distinct ligand components that are recognized by two different CLRs, Mincle and Dectin-2.

Identification of Two Glycolipids as Mincle Ligands

We first tried to identify the Mincle ligand(s) in *Malassezia*. The C:M-soluble fraction was separated into 49 fractions by silica gel column chromatography. Extracts from these fractions showed strong ligand activity that peaked at fractions 44 and 45 (Figure 3A, top). A thin-layer chromatography (TLC) analysis demonstrated that these fractions (44 and 45) contain several spots that were considered to be candidates for Mincle ligands (Figure 3A, bottom). We further analyzed fraction 44 by means of high-performance thin-layer chromatography (HPTLC) and separated it into 20 subfractions to identify the active lipid components. Fraction 44 contained two peaks of ligand activity, 44-1 and 44-2, corresponding to the position of two purple-red spots detected by orcinol staining. These results showed that 44-1 and 44-2 contain glycolipids that are different from TDM (Figure 3B). We further purified 44-1 and 44-2 from fraction 44 by reversed-phase column chromatography and HPLC, and their chemical structures were determined on the basis of the chemical and spectroscopic evidence using fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), proton nuclear magnetic resonance (^{1}H-NMR), carbon NMR (^{13}C-NMR), and gas chromatography-mass spectrometry (GC-MS).

We first analyzed the 44-1 structure (Figure S2A). The negative FAB-MS spectrum of 44-1 showed a pseudomolecular ion peak at *m/z* 919 $[M - H]^-$. The fragment ion peaks due to fatty acid anions were observed at *m/z* 297 $[C_{18}H_{37}COO^-]$ and 241 $[C_{14}H_{29}COO^-]$, as shown in Figure 3C. The molecular formula of 44-1 was further determined to be $C_{49}H_{92}O_{15}$ by the

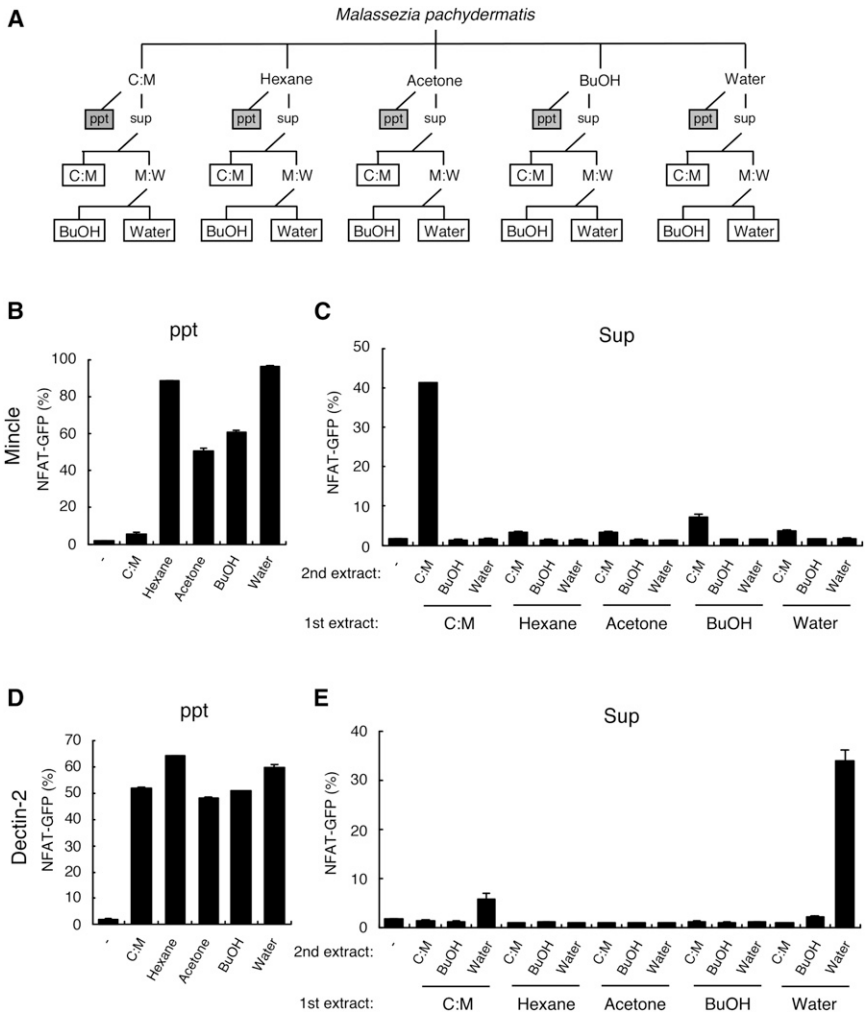

Figure 2. Isolation of Mincle and Dectin-2 Ligands in *Malassezia*

(A) A schematic diagram of the solvent-based fractionation of *M. pachydermatis*. Solvent-treated fungal cells (ppt, gray boxes) and soluble extracts (sup, open boxes) were subjected to reporter assays. C:M, chroloform:methanol.

(B and C) Reporter cells expressing Mincle + FcRγ were stimulated with solvent-treated *M. pachydermatis* cells (B) and soluble extracts in a plate-coated form (C). The NFAT-GFP induction was analyzed by flow cytometry for (B). Data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(D and E) Reporter cells expressing Dectin-2 + FcRγ were stimulated with solvent-treated *M. pachydermatis* cells (D) or soluble extracts in a plate-coated form (E). All data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown. See also Figure S2.

ESI-TOF-MS (*m/z* 943.6362, calculated for 943.6328 $[M + Na]^+$) (Figure S3A). The ^{1}H-NMR spectrum of 44-1 exhibited a strong broad signal due to the aliphatic methylenes at δ_H 1.22, a terminal methyl signal at δ_H 0.80, and several multiplets between δ_H 3.80 and 4.80 due to oxygenated methine and methylene protons. Two typical anomeric proton signals were also detected at δ_H 4.97 (^{1}H, d, J = 7.9 Hz) and δ_H 4.78 (^{1}H, d, J = 7.6 Hz), suggesting two β-linked monosaccharides (Figure S3B). The ^{13}C-NMR spectrum exhibited two terminal methyl signals at δ_C 11.3 and 19.1, aliphatic methylenes at δ_C 25–35, 14 oxygenated methylenes, methines at δ_C 62–78, two anomeric carbon signals at δ_C 104.4 and 104.8, and two estercarbonyl signals at δ_C 173.3 and 173.4. These results indicated that 44-1-2 was a glycerolglycolipid (Figure S3C).

The structure of the sugar and glycerol moiety was determined as follows. The ^{1}H-^{1}H correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra revealed the two independent correlations from H-1 to H-6 of β-glucopyranoses and H-1 to H-3 of a glycerol. The connectivity of two glucopyranoses and a glycerol was determined based on the heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations between the H-1″ (δ_H 4.97) of β-Glc*p* and C-6′ (δ_C 69.6) of β-Glc*p* and between H-1′ (δ_H 4.78) of Glc*p* and C-3 (δ_C 68.1) of glycerol. The terminal structure of (ω-3)-branched fatty acid was confirmed by the chemical shift value and heteronuclear single-quantum correlation spectroscopy (HSQC) correlations of terminal methyl signals (Figures S3D–S3G) (Pan et al., 2010). Composition of the neutral sugar and fatty acids was conducted by GC-MS analysis following methanolysis (Figures S3H–S3J).

Taken together, the less polar glycolipid (44-1) was a glyceroglycolipid with one glycerol, one gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucopyranose), and anteiso-fatty acids C_{14} and C_{18}, which are attached via ester bonds to the hydroxyl groups of the glycerol backbone (Figures 3D and S3K). In addition to 44-1, a small amount of related compounds that are similar to 44-1, except for the length of acyl chains, were identified. We confirmed that all of these compounds possess comparable Mincle ligand activity (Figures S4A and S4B).

Interestingly, 44-1 has a structural similarity to the membrane anchor moiety of lipoteichoic acid (LTA), a bacterial component recognized by TLR2 (Schwandner et al., 1999). However, LTA did not act as a Mincle ligand (Figures S4C–S4E).

We next analyzed the structure of 44-2 (Figure S2B). The positive ESI-TOF-MS spectrum of 44-2 showed a pseudomolecular ion peak at *m/z* 1700.0501 $[M + Na]^+$ and gave the molecular formula as $C_{84}H_{156}O_{32}Na$ (calculated for 1700.0472) (Figure 3E). The ^{1}H-NMR spectrum of 44-2 exhibited a typical spectrum feature due to glycolipids (Figure S3L). Three anomeric proton signals at δ_H 4.96 (^{1}H, brs), 5.00 (^{2}H, brs), and 5.43 (^{1}H, brs) were assignable to β-mannopyranose from the correlations of ^{1}H-^{1}H COSY, TOCSY, nuclear Overhauser effect spectroscopy (NOESY), and HSQC spectra (Figures S3M–S3P). Because the molecular mass of 44-2 was too large to elucidate by only spectroscopic analysis,

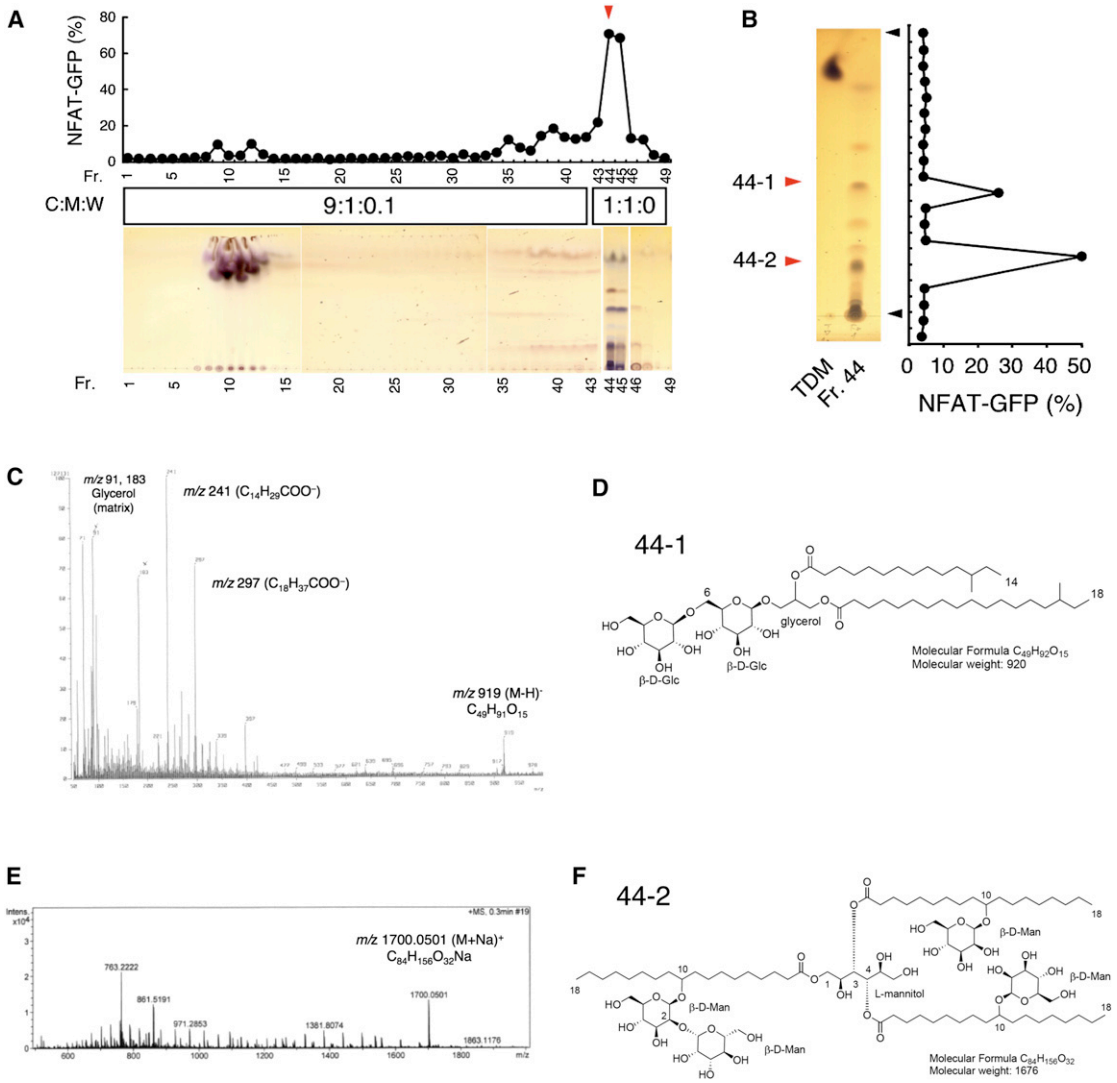

Figure 3. Identification of Mincle Ligands in *Malassezia*

(A) Silica gel column purification. C:M extract of *M. pachydermatis* was subjected to silica gel column chromatography (Silica gel 60; Merck) and eluted with $CHCl_3$/MeOH/H_2O (9/1/0.1 to 1/1/0; v/v/v) to yield 49 fractions. Each fraction was coated onto a plate to stimulate reporter cells expressing Mincle and FcRγ (top). Each fraction was also separated by TLC followed by orcinol staining (bottom). The arrowhead indicates fraction 44.

(B) TLC separation. Fraction 44 (Fr. 44) was analyzed by HPTLC and divided into 20 subfractions. Each subfraction was coated onto a plate to stimulate reporter cells expressing Mincle and FcRγ. The black arrowheads show the origin and solvent fronts. The red arrowheads indicate spots corresponding to 44-1 and 44-2 by orcinol staining. Purified TDM was used as a control.

(C) Mass spectrum of 44-1. The negative FAB-MS spectrum of 44-1 showed a pseudomolecular ion peak at *m/z* 919 $[M - H]^-$. The fragment ion peaks due to fatty acid anions were observed at *m/z* 297 $[C_{19}H_{37}COO^-]$ and 241 $[C_{14}H_{29}COO^-]$.

(D) The chemical structure of Mincle ligand 44-1. See also Figure S3.

(E) Mass spectrum of 44-2. The molecular formula of 44-2 was determined to be $C_{84}H_{156}NaO_{32}$ by the ESI-TOF-MS (*m/z* 1700.0501, calculated for 1700.0472 $[M + Na]^+$).

(F) The chemical structure of Mincle ligand 44-2. See also Figure S3.

the chemical conversion methods were used for structure elucidation. GC-MS analysis following methanolysis clearly revealed that 44-2 consists of D-mannose, mannitol, and 10-hydroxystearic acid (Figures S3Q–S3T). ^{1}H-NMR, TOCSY, HSQC, and HMBC spectra of 44-2 at a lower temperature clarified that the three acyl moieties were linked to the hydroxyl group of the 1,3,4-position of mannitol (Figures S3U–S3X). The treatment of 44-2 with 0.5 M NaOMe yielded two glycosyl fatty acids and L-mannitol (Figures S4F–S4U). The structure of two glycosyl fatty acids were determined to be 10-*O*-β-D-mannopyranosyl stearic acid methyl ester and 10-*O*-β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl stearic acid methyl ester using mass spectrometry and NMR analysis, respectively. Taken together, the analysis on the native form of 44-2 revealed that the polar glycolipid (44-2) has a mannitol backbone, which is attached to two 10-*O*-β-D-mannopyranosyl-10-hydroxy-octadecanoic acids and

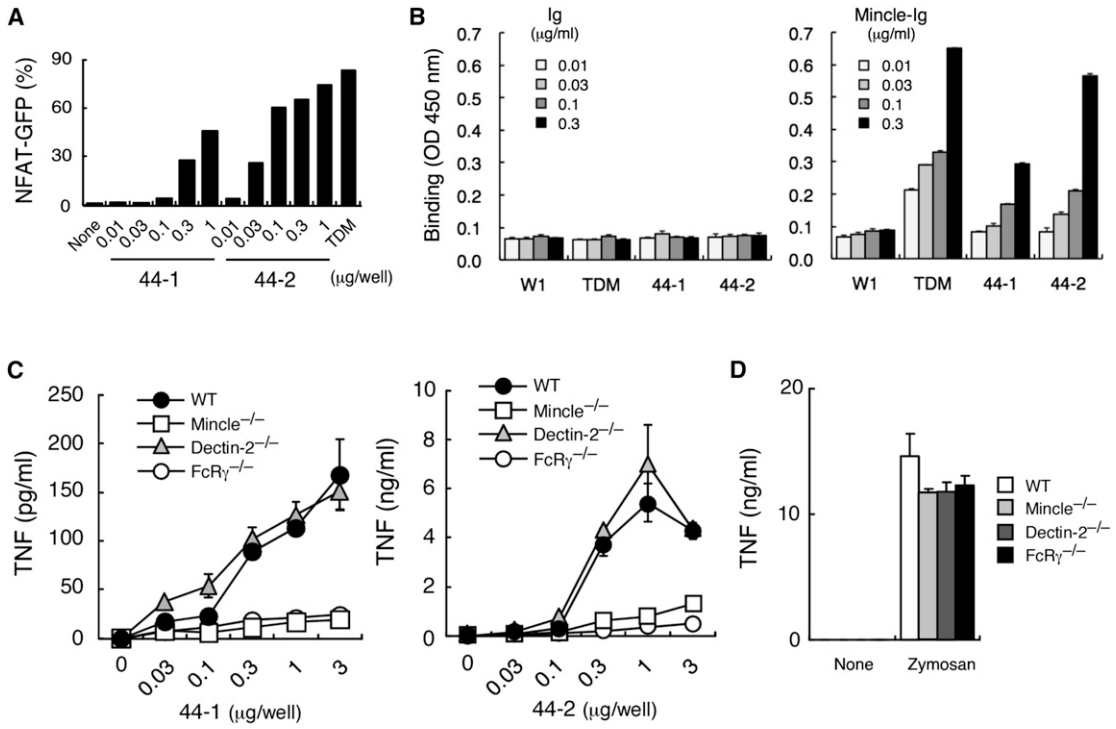

Figure 4. Mincle Is Necessary and Sufficient for the Recognition of Two Glycolipids from *Malassezia*

(A) 44-1 and 44-2 derived from *M. pachydermatis*-activated Mincle-expressing cells. NFAT-GFP reporter cells expressing Mincle + FcRγ were cocultured for 18 hr with plates coated with indicated amount of 44-1, 44-2, or TDM (0.27 μg/well) as a control. See also Figure S4.

(B) Binding of Mincle-Ig fusion protein to 44-1 and 44-2. Serially diluted Ig control (Ig) and Mincle-Ig fusion proteins were allowed to react with 44-1 and 44-2 coated on ELISA plates (1 μg/well). W1 and TDM were used as negative and positive control, respectively. The bound Fc fusion proteins were detected using horseradish peroxidase (HRP)-conjugated anti-hIgG. The data are the means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

(C and D) Essential role of Mincle-FcRγ axis in cytokine production induced by 44-1 and 44-2. BMDCs from WT, FcRγ$^{-/-}$, Dectin-2$^{-/-}$ and Mincle$^{-/-}$ mice were stimulated with plates coated with 44-1 or 44-2 (C) or zymosan (10 μg/ml) as a control (D). The culture supernatants were collected at 48 hr, and their concentrations of TNF were determined by ELISA. The data are the means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

one 10-*O*-(β-D-mannopyranosyl-[1→2]-β-D-mannopyranosyl)-10-hydroxy-octadecanoic acid via ester bonds (Figure 3F).

Thus, 44-2 is a Mincle ligand with a unique structure. Interestingly, only weak activity was detected in glycoside components of 44-2 obtained by alkaline hydrolysis (Figures S4F–S4U), suggesting that the intact form of the 44-2 structure is required for the potent ligand activity.

Mincle Is Necessary and Sufficient for the Recognition of *Malassezia* Glycolipid Ligands

The ligand activity of these glycolipids was verified using Mincle-expressing reporter cells. 44-1 and 44-2 had a Mincle ligand activity as potent as TDM (Figure 4A). We further confirmed that Mincle directly binds to these glycolipids by using soluble Mincle-immunoglobulin (Ig) protein (Figure 4B). These results indicate that *Malassezia* fungi possess two Mincle ligands with unique structures.

To examine the contribution of endogenous Mincle as a receptor for these glycolipids, we tested the ability of 44-1 and 44-2 to activate DCs. Wild-type (WT) bone marrow-derived dendritic cells (BMDCs) were able to secrete tumor necrosis factor (TNF) in response to 44-1 and 44-2 (Figure 4C). This TNF production was almost completely suppressed in Mincle$^{-/-}$ DCs, indicating that Mincle is an essential receptor for 44-1 and 44-2 in DCs (Figure 4C). This finding also confirmed that the observed DC activation is not due to possible contaminating TLR ligands in these fractions, since TLR signaling is intact in Mincle$^{-/-}$ mice (Ishikawa et al., 2009). Dectin-2 was dispensable for the recognition of these glycolipids, which is consistent with the results of reporter cells. FcRγ, a signaling subunit of Mincle, was also essential for the response to the glycolipids, whereas zymosan induced a similar response in all of these cells (Figure 4D). We therefore concluded that Mincle is an essential receptor for the cytokine production induced by two glycolipids derived from *Malassezia*.

Dectin-2 Recognizes *Malassezia* through α-1,2-Linked Mannose

Dectin-2 contains a glutamic acid-proline-asparagine (EPN) motif, which is known to preferentially bind to mannose (Drickamer, 1992). To assess whether the recognition of *Malassezia* cells by Dectin-2 requires this motif, we substituted residues of the EPN motif of Dectin-2 to create a glutamine-proline-aspartic acid (QPD) motif, which is known as a galactose-binding motif

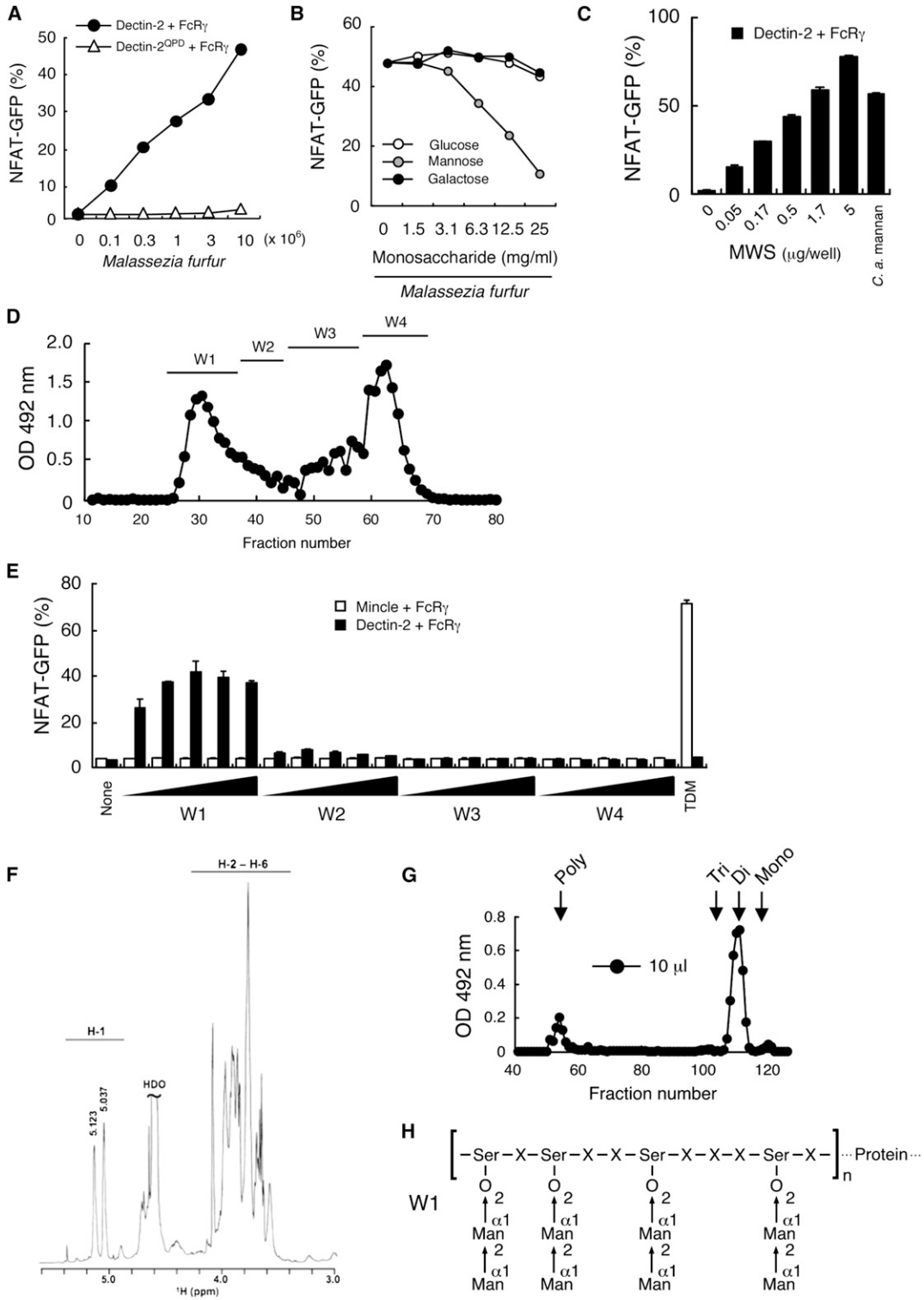

Figure 5. Identification of the Dectin-2 Ligand in *Malassezia*

(A) Role of mannose-binding motif of Dectin-2 in *Malassezia* recognition. NFAT-GFP reporter cells expressing FcRγ together with Dectin-2^{WT} or Dectin-2^{QPD} were cocultured for 18 hr with *M. furfur*. The data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(B) Blocking of *Malassezia* recognition by monosaccharides. NFAT-GFP reporter cells expressing Dectin-2 + FcRγ were cocultured with 3 × 10^6 of *M. furfur* in the presence of glucose, mannose, or galactose. The data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(legend continued on next page)

Table 1. ^{1}H and ^{13}C Chemical Shifts for W1 and Mannobiose Released from W1 by β-Elimination

Mannoprotein or Oligosaccharide	Residue	H-1 ($J_{H1, H2}$)	H-2 ($J_{H2, H3}$)	H-3 ($J_{H3, H4}$)	H-4	H-5	H-6/H-6′
Mannoprotein	→2Manα-*O*-S/T	5.123	3.960	3.783	3.636	–	–
W1	Manα1→	5.037	4.073	3.843	3.652	–	–
β-elimination	→2Man-ol	3.926	3.783	4.021	3.690	3.768	3.877/3.679
Man_2-ol	Manα1→	5.001 (1.8)	3.989 (3.3)	3.871 (9.6)	3.668	3.799	3.908/–
	Residue	C-1	C-2	C-3	C-4	C-5	C-6
Man_2-ol	→2Man-ol	62.07	80.27	68.49	70.40	71.91	63.98
	Manα1→	102.00	71.32	71.20	67.68	74.06	61.81

Sample was dissolved in D_2O. The ^{1}H-NMR spectra were recorded by a JNM-LA600 spectrometer (JEOL) at 45°C. The proton and carbon chemical shifts were referenced relative to the internal acetone at δ 2.225 and 31.07, respectively. W1 was isolated from *M. furfur*. *J* = Hz. Man-ol, mannitol. See also Figure S7 and Table S1.

(Drickamer, 1992). *M. furfur* failed to activate reporter cells expressing Dectin-2 mutant (Dectin-2^{QPD}) (Figure 5A).

To determine the saccharide through which Dectin-2 recognizes *Malassezia*, we tried to block the recognition with various kinds of monosaccharides. An excessive amount of mannose was able to block the NFAT-GFP reporter activity induced by *Malassezia* cells, whereas glucose and galactose did not show any blocking activity (Figure 5B). These results suggest that Dectin-2 may recognize *Malassezia* through a mannose-related structure.

A Hydrophilic Component Has Dectin-2 Ligand Activity

We next searched for the Dectin-2 ligand in *Malassezia*. The *Malassezia* water-soluble fraction (hereafter referred to as MWS) had Dectin-2 ligand activity (Figure 5C). The MWS could activate cells expressing Dectin-2 in amounts as low as 50 ng (Figure 5C), whereas it had no effect on Mincle-expressing cells, even in amounts as high as 5 μg (data not shown). We have reported previously that the cell wall matrix glycoprotein of *Malassezia* contains cell wall β-glucan (Shibata et al., 2009). To enrich the Dectin-2 ligands, we digested β-glucan in MWS with westase (β-1,6-glucanase) because it has not been reported that Dectin-2 recognize β-glucan. We confirmed that the westase digestion of MWS did not impair ligand activity (data not shown).

The reaction product was fractionated by gel filtration chromatography to give four fractions, W1–W4, separated on the basis of their molecular mass (Figure 5D). W1 activated reporter cells expressing Dectin-2, whereas W2, W3, and W4 did not show strong Dectin-2 ligand activity (Figure 5E).

Identification of *O*-Linked Mannoprotein as a Dectin-2 Ligand

Dectin-2 recognizes several fungi that possess *N*-linked mannan on their surface (McGreal et al., 2006; Sato et al., 2006). However, the ^{1}H-NMR analysis of W1 showed only two H-1 signals (Figure 5F and Table 1), suggesting that W1 may possess structure distinct from *N*-linked mannan. The chemical shifts at 5.04 ppm of W1 indicated the presence of a nonreducing terminal α-1,2-linked mannose residue (Shibata et al., 2007).

We further analyzed the structure of W1 by methylation analysis. W1 only has a nonreducing terminal mannose residue and a 2-*O*-substituted mannose residue in the molar ratio of 1:1 (Table S1). These data suggest that W1 may be a mannoprotein possessing predominantly *O*-linked mannooligosaccharides, though such a cell wall matrix glycoprotein has not been identified in yeasts and fungi.

To test this idea, we treated W1 with 0.1 M NaOH to induce β-elimination, which selectively releases *O*-linked oligosaccharides connected to serine and/or threonine (S/T) residues. Bio-Gel P-2 column chromatography of the reaction product showed that about 90% of the carbohydrate was released and eluted only in the disaccharide fraction (Figure 5G). Sugar composition analysis revealed that the eluted disaccharide consists of only mannose (Figure S7A). This result is significantly different from

(C) Activation of Dectin-2-expressing cells by hydrophilic fraction of *M. furfur*. NFAT-GFP reporter cells expressing Dectin-2 + FcRγ were cocultured for 18 hr with plates coated with MWS (0.05–5 μg/well) or *Candida albicans* cell wall mannan (*C. a*. mannan; 0.1 μg/well) as a control. The data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(D) Size fractionation by gel filtration chromatography. Separation of MWS was performed with a column (4.0 × 40 cm) of Sephacryl S-100, and the carbohydrate content in each fraction was assayed by the phenol/sulfuric acid method. Four fractions, W1–W4, were collected on the basis of their molecular mass and subjected to the following assay.

(E) Detection of ligand activity in W1 fraction. NFAT-GFP reporter cells expressing Dectin-2 or Mincle were cocultured for 18 hr with plates coated with W1, W2, W3, and W4 (0.01–1 μg/well) or TDM (0.27 μg/ml/well) as a control. The data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(F) The ^{1}H-NMR analysis of *M. furfur* W1. W1 was dissolved in D_2O. The ^{1}H-NMR spectra were recorded by a JNM-LA600 spectrometer (JEOL) at 45°C. See also Table S2.

(G) Gel filtration chromatography. The elution profiles of β-elimination products of *M. furfur* W1 were shown. Elution was performed with a column (2.5 × 100 cm) of Bio-Gel P-2, and the carbohydrate content in each fraction was assayed by the phenol/sulfuric acid method. The arrows indicate the fraction corresponding to monosaccharide (mono), disaccharide (di), trisaccharide (tri), and polysaccharide (poly). See also Figure S5.

(H) Schematic representation of a possible structure of W1. X is any amino acid.

that of the β-elimination of the mannans from *C. albicans*; the amount of the released oligosaccharides comprises only about 3%–5% of the total carbohydrate (data not shown).

The ^{1}H-NMR and methylation analyses of the released biose fraction indicated that the *O*-linked oligosaccharide was an α-1,2-linked mannobiose, Manα1-2Man (Table 1, Figures S7B and S7C, and Table S1). To study the molar ratio of S/T residues, we investigated the amino acid composition of the protein moiety of mannosyl W1 and found that W1 was abundant in serine (32.9%, Table S2).

On the basis of these results, we concluded that the structure of W1 was a mucin-like serine-rich glycoprotein and the *O*-linked oligosaccharide was predominantly an α-1,2-linked mannobiose (Figure 5H). Thus, although Dectin-2 recognizes *Malassezia* as well as *C. albicans*, the ligand structure of *Malassezia* was quite different from that of *C. albicans*.

Dectin-2 Is Essential for the Cytokine Production Induced by W1

Next, to assess whether Dectin-2 is an essential receptor for the *Malassezia* ligand in DCs, WT, Mincle$^{-/-}$, Dectin-2$^{-/-}$, and FcRγ$^{-/-}$, BMDCs were stimulated with MWS and W1. MWS and W1 were capable of activating WT DCs to produce TNF. However, the TNF production was almost completely suppressed in Dectin-2$^{-/-}$ and FcRγ$^{-/-}$ cells, but not in Mincle$^{-/-}$ DCs (Figure 6A). These findings demonstrated that Dectin-2 is an essential receptor for the *Malassezia*-derived *O*-mannobiose-rich protein, which can directly activate DCs to produce inflammatory cytokines.

Malassezia-Derived Ligands Induce Host Immune Responses

We then examined the effect of these *Malassezia*-derived ligands on immune responses. First, 44-2 and W1 were intraperitoneally (i.p.) injected in mice to assess innate immune responses against these ligands. 44-2 and W1 are capable of inducing neutrophil infiltration into peritoneal cavities (Figure 6B). Furthermore, whole *Malassezia* cells and 44-2 showed adjuvanticity toward acquired immune responses, such as interferon (IFN)γ production, in response to recall antigen stimulation. Although the Mincle ligand is capable of driving interleukin-17 (IL-17)-producing effector T helper cell (Th17) responses (Werninghaus et al., 2009), IL-17 was not detected in our limited condition (Figure S6).

Mincle and Dectin-2 Contribute to Cytokine Production in Response to Whole *Malassezia* Cells

Finally, we investigated the contribution of the two CLRs to the recognition of whole *Malassezia* cells. The production of TNF in response to *M. furfur* was decreased in the absence of Mincle or Dectin-2, thus suggesting that both Mincle and Dectin-2 can mediate DC activation in response to *M. furfur* (Figure 6C). In line with these observations, the TNF production was severely impaired in DCs lacking FcRγ, a common subunit of Mincle and Dectin-2. We found that *M. furfur* also induces IL-10 production, which was also dependent on Mincle and Dectin-2 (Figure 6D). These results suggest that Mincle and Dectin-2 cooperatively contribute to cytokine production in response to the *Malassezia* species.

DISCUSSION

Here we show the identification of distinct ligands for Mincle and Dectin-2 in *Malassezia* fungus.

The general principle of the Mincle ligand structure has not yet been clearly defined. Mincle recognizes TDM and its analog trehalose dibehenate (TDB), but not mycolate or trehalose alone (Ishikawa et al., 2009; Schoenen et al., 2010). Trehalose is a disaccharide formed by two glucoses with an α,α-1,1 linkage. In this study, we found that Mincle ligand 44-1 has one gentiobiose, a disaccharide composed of two units of glucose with a β-1,6 linkage, and two fatty acids, which are attached via ester bonds to the hydroxyl groups of the glycerol backbone. In contrast, 44-2 has two mannosylated fatty acids and one β-1,2-linked mannobiosylated fatty acid, all three of which are attached to the mannitol backbone, although there was minor structural variability such as number of mannose or hydroxyl fatty acid residues. A bipolar glycolipid with disaccharide composed of glucose or mannose attached to fatty acids may represent a potential minimal ligand structure for Mincle. Furthermore, comparisons of the fatty acid moieties of 44-1, 44-2, TDM, and TDB suggest that the length of the fatty acids may not critically influence the Mincle ligand activity.

The reason that Mincle selectively recognizes *Malassezia* among the various fungi remains unclear. Given that *Malassezia* uniquely requires lipid for their growth (Schmidt, 1997), the uptake of exogenous lipid as nutrition may be required for the biosynthesis of long-chain fatty acid moiety of Mincle ligands in fungus. Currently, the roles of 44-1 and 44-2 in the physiology and pathogenesis of *Malassezia* still remain unclear, although it is possible that other glycolipids are synthesized in other *Malassezia* strains.

On the other hand, it has been reported that Dectin-2 recognizes the terminal mannose of *N*-linked glycan (McGreal et al., 2006). In the present study, we demonstrated that *O*-linked mannoprotein could be a Dectin-2 ligand. α-1,2-mannosyl residues of W1 were necessary and sufficient for the recognition by Dectin-2 (Figures S7D and S7E). Although we detected similar *O*-linked mannoprotein in *M. pachydermatis* and *M. sympodialis* (Figure S1B), other *Malassezia* strains may contain different forms of Dectin-2 ligands. Taken together, active Dectin-2 ligand could be defined as a multivalent terminal α-1,2-mannose attached to glycans, proteins, and presumably any kind of scaffold. In line with this hypothesis, *C. guilliermondii* and *S. cerevisiae* were not recognized by Dectin-2 (Figure 1), most likely because the cell wall of these fungi contains α-1,2-mannose masked with β-1,2-mannose and α-1,3-mannose, respectively (Romero et al., 1999; Shibata et al., 1996).

Protein mannosylation is an important process in fungal physiology. It has been reported that protein *O*-mannosyltransferases (PMTs) and α-1,2-mannosyltransferases (MNTs) mediate *O*-mannosylation of proteins in several fungi (Deshpande et al., 2008). Deficiency of these enzymes in several fungi results in an attenuation in their virulence, adherence to host cells, biofilm formation, and cell interaction during mating (Munro et al., 2005; Timpel et al., 1998). Therefore, the *O*-mannosylated products would be appropriate pathogen-associated molecular patterns (PAMPs) for the host to induce an immune response against the fungus. Characterization of W1 core protein by SDS-PAGE

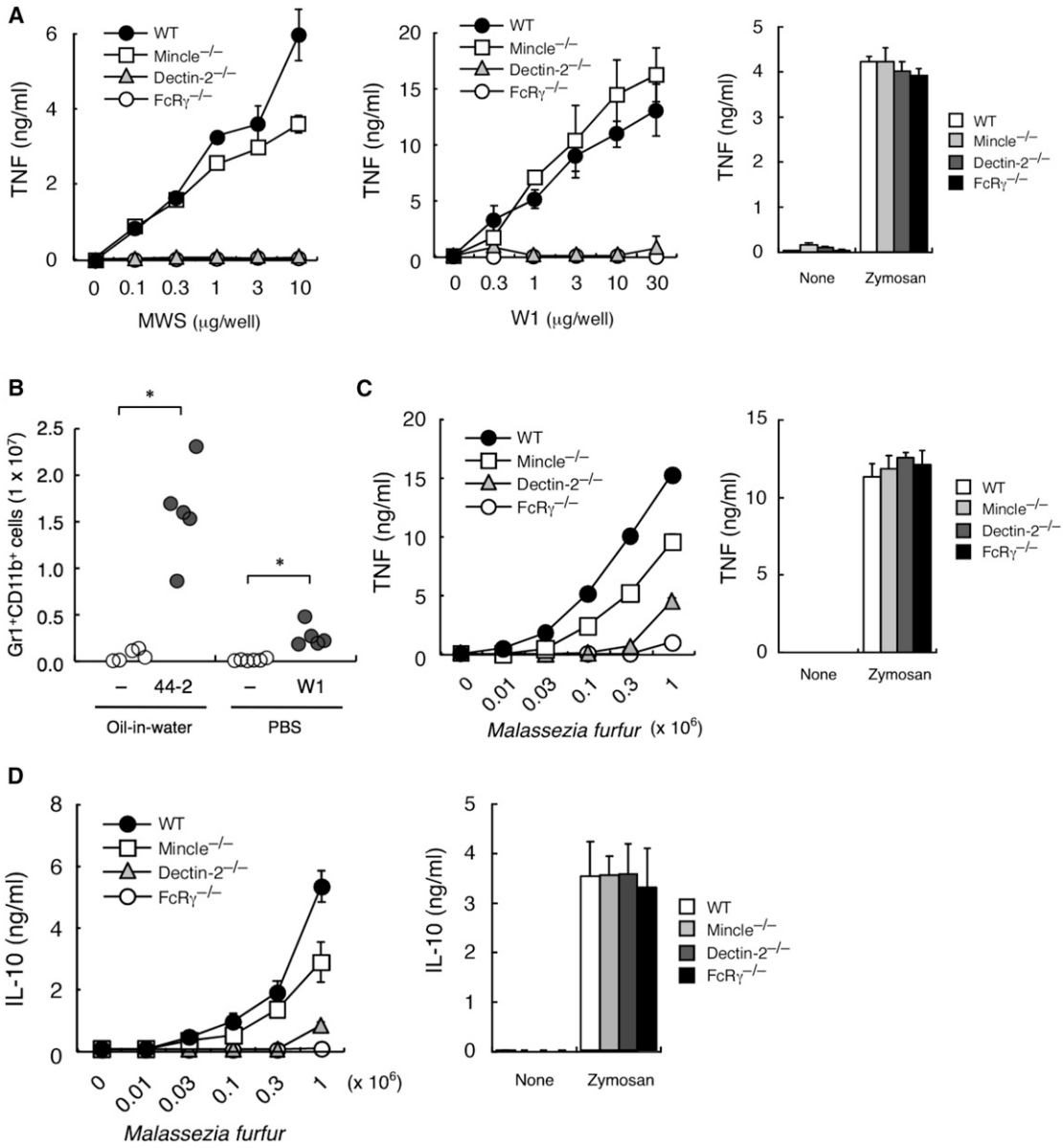

Figure 6. Mincle and Dectin-2 Mediate *Malassezia*-Induced Immune Responses

(A) BMDCs from WT, FcRγ$^{-/-}$, Dectin-2$^{-/-}$, and Mincle$^{-/-}$ mice were stimulated with plates coated with W1 or MWS or zymosan (10 μg/ml) as a control. Culture supernatants were collected at 48 hr, and their concentrations of TNF were determined by ELISA. The data are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(B) Mice were intraperitoneally injected with 200 μg 44-2 in oil-in-water emulsion consisting of mineral oil (9%), Tween 80 (1%), and PBS (90%). Mice were also injected i.p. with 200 μg W1 in PBS. At 20 hr after injection, peritoneal cells were stained with CD11b and Gr1 and analyzed by flow cytometry. Each symbol represents an individual mouse. *$p < 0.05$. See also Figure S6.

(C and D) BMDCs from WT, FcRγ$^{-/-}$, Dectin-2$^{-/-}$, and Mincle$^{-/-}$ mice were stimulated with *M. furfur* or zymosan (10 μg/ml) as a control. After 48 hr of culture, the concentrations of TNF (C) and IL-10 (D) were determined by ELISA. The data are the means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

suggested that apparent molecular mass of protein moiety is approximately 10 kDa (Figure S5). The identification of mannosyltransferase in *Malassezia* may help to clarify the mechanism regulating the biosynthesis of such PAMPs.

Several reports have suggested that *Malassezia* species are associated with atopic dermatitis (Ashbee and Evans, 2002; Scheynius et al., 2002). Many kinds of antigens in *Malassezia* have been demonstrated to react with patient immunoglobulin E (IgE) (Ashbee and Evans, 2002). However, the precise molecular mechanisms underlying the pathogenesis remain unclear. The reactivity of the *O*-mannosyl protein to patient IgE would be an intriguing issue to address.

Malassezia is known to alter the antigens expressed throughout their growth cycle and culture conditions (Ashbee

and Evans, 2002; Shibata et al., 2009). Indeed, it is therefore possible that the relative amount, localization, and structure of Mincle/Dectin-2 ligands in *Malassezia* may also vary according to the life cycle, nutritional status, temperature, or substrains. Taken together, the acquisition of two CLRs recognizing different ligands in the same fungus would enable the host to exert stable immune responses against variable pathogens.

A synthetic LTA anchor, which has structural similarity to 44-1, has been reported to induce TNF production from macrophages in a TLR-independent manner (Morath et al., 2002). Mincle might be a responsible receptor for this response.

Although *Malassezia* is a pathogenic fungus, it is usually a harmless commensal found in healthy skin. It is important to examine whether the expression/function of Mincle/Dectin-2 in langerhans cells or dermal DCs is downregulated in healthy skin. Alternatively, some inhibitory receptors recognizing *Malassezia* may be expressed in such dermal cells to prevent unnecessary DC activation. IL-10, an anti-inflammatory cytokine induced by *Malassezia*, may also play a role in regulating host immune responses to *Malassezia*.

It has recently been revealed that several CLRs recognize the damage-associated molecular patterns (DAMPs) derived from damaged tissue (Aragane et al., 2003; Nauta et al., 2003; Ogden et al., 2001; Oka et al., 1998; Yamasaki et al., 2008; Yuita et al., 2005). On the other hand, terminal mannose residues of glycoproteins become exposed upon inflammation and stress (Franz et al., 2006), although they are normally masked with complex branched sugars during protein maturation in vertebrates (Green et al., 2007). It is therefore possible that Dectin-2 may recognize damaged self through terminal mannose residues of self protein. The identification of endogenous ligand for Mincle, Dectin-2, and other CLRs may help to elucidate the immune responses to damaged tissue through DAMP-PRR interaction.

EXPERIMENTAL PROCEDURES

Mice

Dectin-2-deficient mice and FcRγ-deficient mice on the C57BL/6 background were described previously (Park et al., 1998; Saijo et al., 2010). Mincle-deficient mice, described previously (Yamasaki et al., 2009), were backcrossed for at least nine generations with C57BL/6 mice. All mice were maintained in a filtered air laminar flow enclosure and given standard laboratory food and water ad libitum. All animal protocols were approved by the Committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University.

Fungi

M. pachydermatis (IFM 48586) was grown on agar plates (Wako) or liquid medium with potato dextrose broth (Difco Laboratories) for 5 days at 32°C. *M. furfur* (IFM 52635) was grown in potato dextrose agar supplemented with 100 μl olive oil (Figure 1) or grown in potato dextrose liquid medium supplemented with 1% Tween 80 (Nacalai Tesque) (Figures 6C and 6D) for 5 days at 32°C.

Reagents

TDM, D-glucose, D-mannose, and D-galactose were purchased from Nacalai Tesque. Zymosan (Z4250), LTA (L4015), ovalbumin (OVA; A5503), and α-mannosidase (M7257) were purchased from Sigma-Aldrich. Westase (9095) and *Candida albicans* cell wall mannan (MG001) were obtained from Takara Bio. Other reagents used for chemical analyses are described in Figures S2–S4.

In Vitro Stimulation

To stimulate the cells, TDM was dissolved in C:M (2:1) at 1 mg/ml in isopropanol. Then, these extracts from fungi were added to 96-well plates at 20 μl/well followed by evaporation of the solvent, as described previously (Ishikawa et al., 2009).

In Vivo Stimulation

For innate immune responses, mice were intraperitoneally injected with 44-2 or W1 ligands in oil-in-water emulsion consisting of mineral oil (9%), Tween 80 (1%), and PBS (90%) or in PBS alone, respectively. At 20 hr after injection, peritoneal cells were collected and stained with anti-CD11b and anti-Gr1 monoclonal antibody (mAb) and analyzed by flow cytometry. For acquired immune responses, mice were immunized with 4×10^7 *M. furfur* (i.p.) or 200 μg OVA together with 200 μg *Malassezia*-derived ligands subcutaneously (s.c.). At 7 days after immunization, splenocytes or inguinal lymph node cells were collected and cultured at 3×10^5 cells/200 μl with *Malassezia* antigen or OVA for 72 hr. Cytokine concentrations in the culture supernatants were determined by ELISA.

Cells

2B4-NFAT-GFP reporter cells expressing Mincle, Dectin-2, and Dectin-2^{QPD} mutant (E168Q/N170D) were prepared as previously described (Yamasaki et al., 2008). For BMDC preparation, BM cells were suspended in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and β-mercaptoethanol, plated at a density of 5×10^6 cells/ml in the presence of culture supernatant of MGM-5 (provided by Dr. S. Nagata) as a source of granulocyte macrophage colony-stimulating factor (GM-CSF), and cultured for 6 days at 37°C. For bone marrow-derived macrophages (BMDMs), L929-conditioned medium was used as a source of M-CSF, and adherent cells were used for the in vitro experiments. ELISA kits for TNF, IL-10, IL-4, IFNγ, and IL-17 were purchased from BD Biosciences or R&D Systems.

Preparation of *Malassezia* Lipophilic Fraction

M. pachydermatis was treated with C:M (2:1; v/v), hexane, acetone, 1-butanol (BuOH), or distilled water. The insoluble fractions were collected. The soluble fractions were further partitioned by C:M:W (8:4:3; v/v) into a lower organic phase (C:M) and upper aqueous phase (M:W). The upper aqueous phase (M:W) was further partitioned by 1-butanol:water (1:1; v/v) into an upper butanol phase (BuOH) and a lower aqueous phase (water). Each fraction was resuspended in a volume of 2-propanol relative to the original cell pellet weight and tested as lipid extracts (Morita et al., 2005).

Preparation of MWS

M. furfur cells were washed with deionized water and dehydrated with acetone. The crude cell surface matrix glycoproteins were extracted with deionized water at 120°C for 2 hr. After centrifugation, the soluble extract was dialyzed against running tap water for 2 days and then evaporated, lyophilized, and used as MWS.

Preparation of W1

MWS was dissolved in 25 ml of 100 mM McIlvain buffer (pH 6.0), and 50 units of westase were added and incubated at 37°C for 24 hr. The enzyme was inactivated by heating at 100°C for 5 min, and the supernatant was separated by centrifugation at 3,000 rpm for 10 min followed by evaporation. The westase reaction product was applied onto a column (4.0 × 40 cm) of Sephacryl S-100 and eluted with deionized water to yield four fractions: W1, W2, W3, and W4. The amount of W1 was approximately 0.5% of the dried cell mass.

Chemical Analysis

FAB-MS, ESI-TOF-MS, GC-MS, and nuclear magnetic resonance spectroscopy (^{1}H-NMR and ^{13}C-NMR) were performed as described in Figures S3 and S4 and Table S1.

Release of *O*-Linked Oligosaccharides from W1 by β-Elimination

W1 (20 mg) was dissolved in 0.5 M $NaBH_4$/0.1 M NaOH and incubated at 25°C for 18 hr. The reaction mixture was neutralized with acetic acid and repeatedly evaporated with methanol to remove boric acid. The reaction mixture was

dissolved in 1 ml of water, applied onto a column (2.5 × 100 cm) of Bio-Gel P-2, and eluted with water. The released oligosaccharide was analyzed by ^{1}H-NMR and the methylation analyses.

Carbohydrate Composition Analysis

For the analysis of the carbohydrate composition, samples were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 100°C for 3 hr. The resulting monosaccharide mixtures were reduced by treatment with $NaBH_4$ and acetylated by acetic anhydride/pyridine (1:1; v/v). The reagents were evaporated and analyzed by GC-MS.

Monosaccharide Linkage Analysis

The methylation analysis was carried out according to the method reported by Ciucanu and Kerek (1984). The mannoprotein or oligosaccharide was dissolved in a NaOH/dimethylsulfoxide suspension prepared using powdered NaOH. After stirring for 30 min, methyl iodide was added, and the suspension was stirred for another 30 min. The methylated product was extracted into chloroform and washed with water. The permethylated carbohydrates were then hydrolyzed in 2 M trifluoroacetic acid at 110°C for 2 hr. The partially methylated monosaccharides were reduced with 1% $NaBD_4$ at room temperature for 18 hr. Following borate removal by drying from methanol, the partially methylated alditols were acetylated by adding acetic anhydride/pyridine (1:1; v/v) and incubating them at 50°C for 3 hr. The reagents were evaporated and analyzed by GC-MS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.03.008.

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